Available online at www.sciencedirect.com

ScienceDirect

Virology 360 (2007) 247–256

VIROLOGY

www.elsevier.com/locate/yviro

Stoichiometry of the antiviral protein APOBEC3G in HIV-1 virions

Hongzhan Xu [a], Elena Chertova [b], Jianbo Chen [c], David E. Ott [b], James D. Roser [b], Wei-Shau Hu [c], Vinay K. Pathak [a,*]

[a] *Viral Mutation Section, HIV Drug Resistance Program, Center for Cancer Research, National Cancer Institute-Frederick, P. O. Box B, Bldg. 535, Rm. 334, Frederick, MD 21702, USA*

[b] *AIDS Vaccine Program, SAIC-Frederick, Inc., National Cancer Institute-Frederick, Frederick, MD 21702, USA*

[c] *Viral Recombination Section, HIV Drug Resistance Program, National Cancer Institute-Frederick, Frederick, MD 21702, USA*

Received 24 March 2006; returned to author for revision 1 May 2006; accepted 17 October 2006
Available online 28 November 2006

Abstract

A host cytidine deaminase, APOBEC3G (A3G), inhibits replication of human immunodeficiency virus type 1 (HIV-1) by incorporating into virions in the absence of the virally encoded Vif protein (Δ*vif* virions), at least in part by causing G-to-A hypermutation. To gain insight into the antiretroviral function of A3G, we determined the quantities of A3G molecules that are incorporated in Δ*vif* virions. We combined three experimental approaches-reversed-phase high-pressure liquid chromatography (HPLC), scintillation proximity assay (SPA), and quantitative immunoblotting-to determine the molar ratio of A3G to HIV-1 capsid protein in Δ*vif* virions. Our studies revealed that the amount of the A3G incorporated into Δ*vif* virions was proportional to the level of its expression in the viral producing cells, and the ratio of the A3G to Gag in the Δ*vif* virions produced from activated human peripheral blood mononuclear cells (PBMC) was approximately 1:439. Based on previous estimates of the stoichiometry of HIV-1 Gag in virions (1400–5000), we conclude that approximately 7 (±4) molecules of A3G are incorporated into Δ*vif* virions produced from human PBMCs. These results indicate that virion incorporation of only a few molecules of A3G is sufficient to inhibit HIV-1 replication.
Published by Elsevier Inc.

Keywords: APOBEC3G virion incorporation; Peripheral mononuclear blood cells; Cytidine deamination; Scintillation proximity assay; Δ*vif* virions

Introduction

High rates of G-to-A substitutions were first observed to occur in retroviral genomes in a spleen necrosis virus and named hypermutation by the authors (Pathak and Temin, 1990); later, G-to-A hypermutations were observed in HIV-1 genomes (Vartanian et al., 1991). Recently, a host cytidine deaminase APOBEC3G (A3G) was identified to be responsible for inhibition of replication of Δ*vif* virions in restricted cell lines (Sheehy et al., 2002). Subsequently, A3G was found to induce G-to-A hypermutations in HIV-1 genomes (Lecossier et al., 2003; Mangeat et al., 2003; Zhang et al., 2003). During reverse transcription, A3G deaminates deoxycytidines in minus-strand DNA to form deoxyuridine, resulting in G-to-A hypermutation and viral inactivation (Harris et al., 2003; Mangeat et al., 2003; Zhang et al., 2003). Although hypermutation is thought to be responsible for viral inactivation, a recent report showed that the antiviral activity of A3G could be independent of its cytidine deaminase activity (Newman et al., 2005), indicating that the mechanism of its antiviral activity may be more complex than previously believed. A3G expression in the virus producer cells results in its incorporation into HIV-1 and this virion packaging plays an important role in A3G's antiretroviral function (Kao et al., 2003; Marin et al., 2003). A3G is an RNA-binding protein, and we as well as other groups recently showed that it is packaged into HIV-1 virions through its interactions with RNA (Svarovskaia et al., 2004; Zennou et al., 2004), whereas other groups have suggested that interactions between A3G and NC are essential for virion incorporation (Alce and Popik, 2004; Cen et al., 2004; Luo et al., 2004; Schafer et al., 2004). HIV-1 encodes Vif, which can overcome the antiviral activity of A3G by inducing its proteolysis through the proteasomal degradation pathway, thereby preventing A3G packaging into virions (Conticello et al., 2003; Kao et al., 2003; Mariani et al., 2003;

* Corresponding author. Fax: +1 301 846 6013.
E-mail address: vpathak@ncifcrf.gov (V.K. Pathak).

0042-6822/$ - see front matter. Published by Elsevier Inc.
doi:10.1016/j.virol.2006.10.036

Marin et al., 2003; Stopak et al., 2003; Yu et al., 2003). In the absence of Vif, the endogenous A3G is packaged into Δ*vif* virions and the replication of these virions is suppressed (Sheehy et al., 2002).

Determination of the amount of A3G packaged in Δ*vif* virions may shed light on the mechanism(s) by which it inhibits HIV-1 replication. While G-to-A hypermutation is generally thought to inactivate viral genomes through lethal mutagenesis, the presence of uridines in the minus-strand DNA has also been shown to inhibit initiation of plus-strand DNA synthesis (Klarmann et al., 2003). A recent study also showed that A3G inhibited viral DNA synthesis independent of its cytidine deamination activity (Bishop et al., 2006; Newman et al., 2005). Most studies of A3G's effects on viral infectivity are performed using transient transfection of A3G-expressing plasmids, and may be complicated by over-expression of A3G in the virus producer cells compared to the levels of A3G that are likely to exist in the natural targets of HIV-1 infection. Overloading of A3G protein in virions could result in inhibition of viral replication through mechanisms that do not operate during natural HIV-1 infection. Therefore, determining the amounts of A3G incorporated into Δ*vif* virions and comparing these levels to those found in virions produced from transfected cells could provide insight into the mechanisms by which A3G inhibits HIV-1 replication. Determining the stoichiometry of A3G in Δ*vif* virions may also provide insights into the nature of interactions between A3G and viral RNA or proteins, whether A3G acts catalytically or as a physical barrier to reverse transcription, and the efficiency with which Vif must prevent its incorporation into virions.

The results of our studies using reversed-phase high-pressure liquid chromatography (HPLC), quantitative immunoblotting, and cytidine deaminase assays indicate that the A3G levels in Δ*vif* virions are approximately 0.228% of HIV-1 Gag, providing an A3G:Gag ratio of 1:439. Thus, based on current estimates of HIV-1 Gag molecules per virion (1400–5000) (Briggs et al., 2004; Layne et al., 1992; Zhu et al., 2003), incorporation of very few enzymatically active A3G molecules (7±4) is sufficient to potently inhibit HIV-1 replication.

Results

Quantitative HPLC determination of A3G:Gag ratio in Δvif virions

To determine the A3G:Gag ratio in Δ*vif* virions, HPLC was used to separate and quantify virion proteins (Fig. 1). HPLC allowed viral proteins to be isolated, characterized, and quantified in one combined procedure, providing accurate measurement of the protein composition of the virus. pHDV-

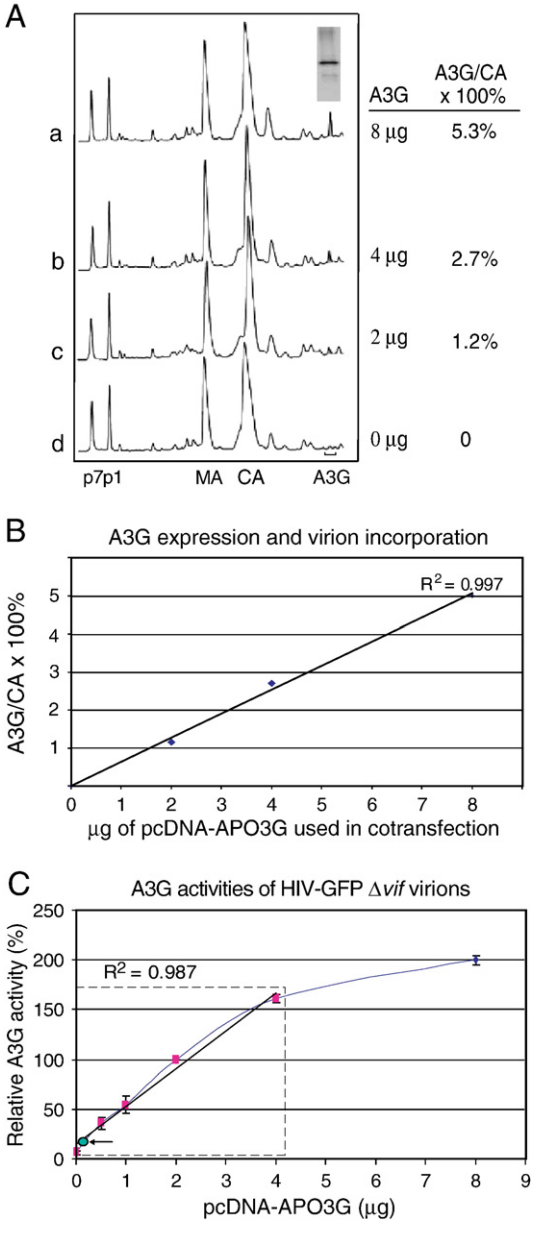

Fig. 1. HPLC analysis and cytidine deaminase activities of HIV-GFPΔ*vif* virions produced from 293T cells cotransfected with 0, 2, 4, or 8 µg of pcDNA-APO3G and from activated $CD4^+$T cells. Virion preparation is described in Materials and methods. (A) Protein elution profiles detected at 280 nm. Proteins eluted from the column of acetonitrile gradient were analyzed by SDS-PAGE to confirm the complete separation by HPLC; the identities of the proteins were confirmed by protein sequencing, immunoblot analysis and amino acid analysis. An example of the immunoblotting analysis performed to confirm the identity of the peak containing A3G is shown in the inset. Peak names are shown at the bottom of the HPLC profiles. After isolation and quantitation of virion-associated A3G and p24 CA, the A3G to CA molar ratio was determined. (B) Correlation between A3G protein incorporated into HIV-GFPΔ*vif* virions and the amount of pcDNA-APO3G cotransfected. The A3G to CA molar ratios as a % of CA are plotted on the *y* axis, and the amount of pcDNA-APO3G used in cotransfection during production of HIV-GFPΔ*vif* virions are plotted on the *x*-axis. (C) Relative A3G activities in HIV-GFPΔ*vif* virions. HIV-GFPΔ*vif* virions harvested from 293T cells cotransfected with 8, 4, 2, 1, 0.5 and 0 µg of pcDNA-APO3G and from three preparations of human $CD4^+$T cells were analyzed, and the relative A3G activities in virions containing 1 ng of p24 CA were determined. The A3G activity of HIV-GFPΔ*vif* virions produced from 293T cells transfected with 2 µg pcDNA-APO3G was set as 100%. The linear portion of the standard curve is shown in a rectangle of dashed lines. Linear regression analysis indicating the best fit is shown as a solid black line, and the R^2 value is indicated. The average relative A3G activity of 1 ng p24 CA of HIV-GFPΔ*vif* virions harvested from human $CD4^+$T cells is represented by a green dot labeled with an arrow.

EGFP is an HIV-1 vector that expresses HIV-1 Gag and Pol proteins as well as EGFP, but does not express Vif, Vpr, Vpu, Env, or Nef proteins. Cotransfection of 293T cells with pHDV-EGFP and 8, 4, or 2 μg of pcDNA-APO3G, a plasmid that expresses A3G, resulted in the production of HIV-GFPΔ*vif* virions containing A3G, which were isolated by density ultracentrifugation, lysed, and subjected to HPLC separation. The HPLC fractions containing p24 CA and A3G proteins were identified by silver staining and immunoblotting; subsequently, the identified fractions were subjected to mass spectrometry and protein sequence analyses. The result showed that the height of the A3G peak was directly proportional to the amount of pcDNA-APO3G used in each cotransfection. In addition, consistent with the very low expression levels of A3G in 293T cells, A3G was not detectable in HIV-GFPΔ*vif* virions produced from 293T cells in the absence of cotransfection with pcDNA-APO3G. To obtain an accurate ratio of the amount of A3G and Gag in HIV-GFPΔ*vif* virions produced from cells transfected with 8 μg of pcDNA-APO3G, we subjected a portion of the corresponding HPLC fractions to automated amino acid analysis. The precise amount of the protein in the sample can be determined by comparing it to a set of known standards. The results of amino acid analysis with reference amino acid standards revealed that the average value for the Gag proteins (average of MA, CA, NC, and p6) was 206 nanomoles and the value for A3G was 11 nanomoles in this sample, indicating that the moles of A3G were 5.3% of the moles of CA, which is equivalent to the A3G:Gag ratio. In addition, the amino acid stoichiometry observed closely matched the expected compositions, indicating that both A3G and CA samples were predominantly pure. Because UV absorbance is directly proportional to the amount of protein, we determined the amount of A3G and CA protein in the 4 and 2 μg A3G cotransfection samples by comparing the integrated peak heights of the CA and A3G fractions of these samples to those corresponding peaks observed for the HIV-GFPΔ*vif* virion samples produced from cells transfected with 8 μg of pcDNA-APO3G. These quantitations were used to calculate the A3G:Gag molar ratios for each HIV-GFPΔ*vif* virion sample (Fig. 1). The results showed that the moles of A3G in HIV-GFPΔ*vif* virions produced from 8, 4 and 2 μg of pcDNA-APO3G cotransfected 293T cells were 5.3%, 2.7%, and 1.2%, respectively, of the moles of Gag (Fig. 1A). The results also indicated that the amounts of A3G in HIV-GFPΔ*vif* virions were directly proportional to the amounts of cotransfected pcDNA-APO3G (Fig. 1B).

Quantitation of A3G activity in HIV-GFPΔvif virions produced from primary CD4$^+$T cells

We sought to determine whether the A3G activities in HIV-GFPΔ*vif* virions correlated with the A3G protein levels determined by HPLC analysis, and the relative A3G enzymatic activities in primary CD4$^+$T cell-derived and 293T-derived HIV-GFPΔ*vif* virions. We quantified the A3G enzymatic activities HIV-GFPΔ*vif* virions by using an A3G-specific scintillation proximity assay (SPA) that we reported previously (Svarovskaia et al., 2004). The assay uses an oligonucleotide substrate that is specific for A3G but not APOBEC3F (Harris and Liddament, 2004) and is unable to detect cytidine deaminase activity from cells transfected with human APOBEC3F, human APOBEC3B, and murine APOBEC3 (data not shown). Thus, the assay primarily measured the cytidine deamination activity of A3G in the samples tested.

The amounts of primary CD4$^+$T cell-derived and 293T cell-derived HIV-GFPΔ*vif* virions were determined by quantifying the amounts of p24 CA protein by ELISA, and the A3G activities associated with HIV-GFPΔ*vif* virions containing 1 ng of p24 CA were compared. Consistent with the HPLC analysis (Fig. 1A), the A3G enzymatic activities in the HIV-GFPΔ*vif* virions produced from these cells were proportional to the amounts of pcDNA-APO3G used for transfection (Fig. 1C). Linear regression analysis of the A3G activities observed for cells transfected with 4, 2, 1, and 0.5 μg of pcDNA-APO3G showed an excellent fit with an R^2 value of 0.987. The primary CD4$^+$T cell-derived HIV-GFPΔ*vif* virions contained an average of 15.7% of the A3G enzymatic activity present in the HIV-GFPΔ*vif* virions produced from 293T cells transfected with 2 μg of pcDNA-APO3G (Table 1). The A3G activities in the primary CD4$^+$T cell-derived HIV-GFPΔ*vif* virions were also significantly higher than the mock-infected negative controls (Table 1; $P<0.001$).

Quantitative immunoblotting analysis of A3G in Δvif virions produced from human PBMCs

We sought to determine the A3G:Gag ratio in virions produced in the presence of all viral proteins except Vif to rule out the possibility that the absence of viral proteins Vpr, Vpu, Env, and Nef influenced A3G packaging in HIVΔ*vif* virions. We used NL43Δ*vif*, an HIV-1 genome that expresses all viral proteins except Vif, to produce NL43Δ*vif* virions from PBMCs and compared the amounts of A3G incorporated to those present in HIV-GFPΔ*vif* virions produced from 293T cells transfected with 2 μg of pcDNA-APO3G. Although the HPLC analysis provided an accurate measurement of the A3G:Gag ratios, the method required large quantities of virions (Zhu et al., 2003). Because it was impractical to produce such large quantities of virions from activated human PBMCs, we used quantitative immunoblotting analysis, a method requiring >10-fold less virions, to compare the amounts of A3G protein in Δ*vif* virions produced from the human PBMCs and transfected 293T cells. To obtain an accurate comparison, the lysed HIV-GFPΔ*vif* virion preparation obtained from 293T cells transfected with pcDNA-APO3G were serially diluted to contain 50, 25, 12.5, and 6.25 ng of p24 CA and compared to PBMC-derived NL43Δ*vif* virion preparations containing 100 ng of p24 CA (Fig. 2A). We compared the 293T-derived and PBMC-derived A3G bands of similar intensities to maximize accuracy of quantitation. The amount of A3G protein in NL43Δ*vif* virions containing 100 ng of p24 CA produced a band that was of similar intensity to the band produced from 293T-derived virions containing 25 ng of p24 CA (Fig. 2B, bands indicated by

Table 1
Relative A3G activities in HIV-GFPΔ*vif* virions sample

Cells	pcDNA-APO3G[a]	Viruses	p24 CA	No. of experiments	Relative A3G activity (%) Average[b]±S.E.	*t* test[c] *P* value	[V-A3G$_{CD4+T}$]/[V-A3G$_{2\ \mu g}$][d]
293T cells	8 μg	HIV-GFPΔ*vif*	1 ng	3	199.7±4.5		
	4 μg		1 ng	3	161.1±4.4		
	2 μg		1 ng	3	100±0.0		
	1 μg		1 ng	3	54.1±8.5		
	0.5 μg		1 ng	3	35.8±6.0		
	0		1 ng	3	6.1±2.0		
CD4$^+$T cell donor #1		HIV-GFPΔ*vif*	1 ng	3	15.7±4.0	<0.001	15.7%
		Mock	Equal vol.[e]	3	6.8±3.0		
CD4$^+$T cell donor #2		HIV-GFPΔ*vif*	1 ng	3	15.7±0.1	<0.001	15.7%
		Mock	Equal vol.	3	6.4±2.0		
CD4$^+$T cell donor #3		HIV-GFPΔ*vif*	1 ng	3	15.7±5.0	<0.001	15.7%
		Mock	Equal vol.	3	8.2±2.0		
Average [V-A3G$_{CD4+T}$]/[V-A3G$_{2\ \mu g}$] ratio							15.7%

[a] Amount of pcDNA-APO3G used to transfect 293T cells.
[b] A3G activity in virions harvested from 2 μg pcDNA-APO3G transfected 293T was set as 100%. The average cpm for samples containing 1 ng p24 CA of HIV-GFPΔ*vif* virions harvested from 293T cells transfected with 2 μg of pcDNA-APO3G was 19,385; the average cpm for samples without any HIV-GFPΔ*vif* virions was 50.
[c] The *t* tests were performed to compare A3G activities in viruses produced from human CD4$^+$T cells with A3G activities in culture supernatant (mock infected sample) of the CD4$^+$T cells from the same donor.
[d] The[V-A3G$_{CD4+T}$]/[V-A3G$_{2\ \mu g}$] ratio was defined as the ratio of A3G activities in ng p24 CA of CD4$^+$T cell-derived HIV-GFPΔ*vif* virions and the A3G activities in 1 ng of p24 CA of HIV-GFPΔ*vif* virions produced from 293T cells transfected with 2 μg of pcDNA-APO3G.
[e] Because the mock infected samples did not contain p24 CA (data not shown), volumes of these samples equivalent to the volume of culture supernatant from PBMCs producing NL43Δ*vif* and containing 1 ng of p24 CA were used in the SPA.

arrows). Quantitation of the band intensities was performed and the A3G band produced with 293T-derived HIV-GFPΔ*vif* virions containing 100 ng of p24 CA was set to 100%. The A3G protein band intensities of the PBMC-derived NL43Δ*vif* virions [67%/100 ng] and the 293T-derived HIV-GFPΔ*vif* virions [75%/25 ng] were used to calculate that the PBMC-derived NL43Δ*vif* virions contained approximately 22% of the A3G present in 293T-derived HIV-GFPΔ*vif* virions (see Fig. 2B, legend). Thus, the amounts of A3G packaged in Δ*vif* virions were proportional to the amounts of A3G expressed in the virus producer cells. In contrast to the NL43Δ*vif* virions, the wild type NL43 virions contained much lower quantities of A3G (13% vs. 67% per 100 ng of p24 CA). This observation confirmed that expression of the Vif protein from the wild type NL43 substantially reduced the amount of A3G that was incorporated into the PBMC-derived virions.

Quantitation of A3G enzymatic activity in NL43Δvif virions produced from human PBMCs

Next, we compared the A3G activities present in NL43 and NL43Δ*vif* virions produced from activated PBMCs to those from 293T cells transfected with 2 μg of pcDNA-APO3G (Table 2 and Fig. 2B). Similar to the analysis of A3G activities in cell lysates, we generated a standard curve by measuring A3G activities in serial dilutions of HIV-GFPΔ*vif* virions produced from 293T cells transfected with 2 μg of pcDNA-APO3G and 20 μg pHDV-EGFP. The standards consisted of HIV-GFPΔ*vif* virion lysates containing 1, 0.5, 0.25 and 0.125 ng of p24 CA harvested from the cotransfected 293T cells; the negative control contained 2 ng of p24 CA from HIV-GFPΔ*vif* virions produced from 293T cells in the absence of pcDNA-APO3G. The A3G activity in the HIV-GFPΔ*vif* viral lysate containing 1 ng of p24 CA was set to 100% (Table 2 and Fig. 2B). The results of SPA showed that the relative A3G enzymatic activities observed in 293T-derived HIV-GFPΔ*vif* lysates containing 1, 0.5, 0.25, and 0.125 ng of p24 CA were proportional to the amount of viral proteins used in the assay. Linear regression analysis of these activities indicated an excellent fit and provided an R^2 value of 0.996 (dashed box in Fig. 3B and Table 2). The HIV-GFPΔ*vif* virion lysates containing 2 ng of p24 CA produced from control 293T cells contained only 4.2% of the A3G activity observed in 1 ng of p24 CA from HIV-GFPΔ*vif* virions produced in the presence of pcDNA-APO3G (Table 2).

We then determined the A3G activities present in three preparations of PBMC-derived NL43 and NL43Δ*vif* virions containing 2 ng of p24 CA and compared these activities to those present in 293T-derived HIV-GFPΔ*vif* virions. The A3G activities in NL43Δ*vif* virion lysates containing 2 ng of p24 CA ranged from 22.9 to 27.7% of the activities in 293T-derived HIV-GFPΔ*vif* virion lysates containing 1 ng of p24 CA (Table 2, samples labeled NL43Δ*vif* and Fig. 2B, green dot labeled with arrow). The A3G activities in the PBMC-derived NL43Δ*vif* virions were within the linear range of the assay (Fig. 2B). The ratio of A3G activity in PBMC-derived NL43Δ*vif* virions [V-A3G$_{PBMC}$] to the A3G activity in HIV-GFPΔ*vif* virions produced from 293T cells transfected with 2 μg of pcDNA-APO3G [V-A3G$_{2\ \mu g}$] was determined by using the activities observed in 0.25 ng of p24 CA from the 293T-derived virions, because these activities were within the linear range and closest to the activities in the PBMC-derived virions. The average [V-A3G$_{PBMC}$]/[V-A3G$_{2\ \mu g}$] ratio was 19.0% (Table 2). Thus, the A3G activity in PBMC-derived NL43Δ*vif* virions

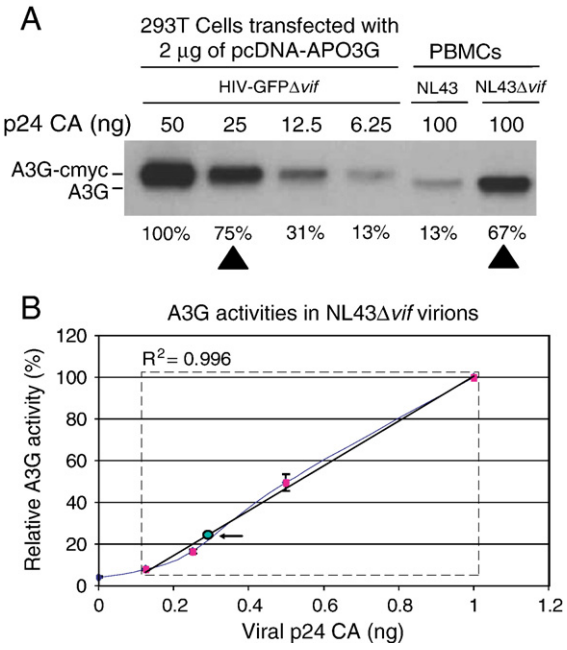

Fig. 2. Quantitative immunoblotting analysis and cytidine deaminase activities of A3G in HIV-1 virions. (A) Quantitative immunoblotting analysis. HIV-GFPΔ*vif* virions were produced from 293T cells cotransfected with 2 μg of pcDNA-APO3G and 20 μg of pHDV-EGFP; NL43 and NL43Δ*vif* virions were produced from PBMCs. The samples contained 50, 25, 12.5 and 6.25 ng of p24 CA of 293T derived HIV-GFPΔ*vif* virions and 100 ng p24 CA of NL43 and NL43Δ*vif* virions produced from PBMCs. The A3G protein was detected using an anti-A3G polyclonal antibody and the intensities of the bands were quantified by QuantityOne software (Bio-Rad). The intensity of the A3G band detected from 293T-derived HIV-GFPΔ*vif* virions containing 50 ng of p24 CA was set to 100%. The amount of A3G protein in the PBMC-derived NL43Δ*vif* virions as a percentage of the amount of A3G in the 293T-derived HIV-GFPΔ*vif* virions was calculated using the bands with similar intensities (indicated by arrows) as follows: 67% A3G/100 ng of PBMC-derived p24 CA/75% A3G/25 ng of 293T-derived p24 CA × 100% = 22%. (B) Determination of A3G activities. in Δ*vif* virions. Standards contained 1, 0.5, 0.25 and 0.125 ng p24 CA of HIV-GFPΔ*vif* virions harvested from 293T cells transfected with 2 μg pcDNA-APO3G. The linear portion of the standard curve is shown in a rectangle of dashed lines. Linear regression analysis indicating the best fit is shown as a solid black line, and the R^2 value is indicated. The average relative A3G activity in 2 ng of p24 CA of NL43Δ*vif* virions harvested from human PBMCs is shown as a green dot labeled with an arrow.

was similar to their A3G protein level (22%) as determined by quantitative immunoblotting (Fig. 2A).

We also determined the A3G activities in PBMC-derived wild type NL43 virions (Table 2). The A3G activities in PBMC-derived NL43 virion lysates containing 2 ng of p24 CA ranged from 6.2 to 7.2% of the A3G activity in 293T-derived HIV-GFPΔ*vif* virion lysates containing 1 ng of p24 CA, indicating that expression of Vif from wild type NL43 in the PBMCs significantly reduced the A3G activity in the virions compared to the A3G activities in the NL43Δ*vif* virions. Finally, we determined the A3G activity present in culture supernatants of mock-infected samples (Table 2). Volumes of culture supernatants equivalent to those containing 2 ng of p24 CA in NL43Δ*vif* samples were used to determine the A3G activities; in equivalent volumes of culture supernatants, the A3G activities in the mock-infected samples ranged from 5.2 to 7.4% compared to the activities present in 293T-derived HIV-GFPΔ*vif* virion lysates containing 1 ng of p24 CA (Table 2). Therefore, the A3G activities in NL43Δ*vif* virions were approximately three to fourfold higher than the wild type NL43 virions and the mock-infected samples.

Quantitative immunoblotting analysis of A3G expression in human PBMCs

Because the HPLC analysis indicated that A3G expression levels in the virus producer cells were directly proportional to the amounts of A3G incorporated into virions, we sought to determine the amounts of A3G in human PBMCs, the natural target cells of HIV-1 infection. We used quantitative immunoblotting analysis to compare A3G expression levels in activated human PBMCs to those in 293T cells transfected with 2 μg pcDNA-APO3G (Fig. 3A). To obtain an accurate comparison, the lysates obtained from 293T cells transfected with pcDNA-APO3G were serially diluted to contain 1, 0.5, 0.25, and 0.125 μg of total protein and compared to 1 and 2 μg of cell proteins obtained from activated PBMCs. In addition, because of the relatively narrow linear dynamic range of Western blotting analysis, we compared the 293T-derived and PBMC-derived A3G bands of similar intensities to maximize accuracy of quantitation. The amount of A3G protein in 1 μg of PBMC protein produced a band that was of similar intensity to the band produced in 0.25 μg of 293T cell protein (Fig. 3A, bands indicated by arrows). Quantitation of the band intensities was performed and the A3G band produced with 1 μg of 293T cell protein was set to 100%. The A3G protein band intensities in the activated human PBMCs [43%/1 μg] and the transfected 293T cells [36%/0.25 μg] were used to determine that the PBMCs contained approximately 30% of the A3G present in 293T cells transfected with 2 μg of pcDNA-APO3G (see Fig. 2A, legend).

Quantitation of A3G enzymatic activity in human PBMCs

In order to accurately measure the A3G activity in human PBMCs, we generated a standard curve by serially diluting total cellular protein harvested from the 293T cells transfected with 2 μg pcDNA-APO3G and 20 μg pHDV-EGFP (Table 3 and Fig. 3B). The standards contained 1.8, 0.9, 0.45, 0.225, 0.113 and 0.056 μg of total protein from the transfected 293T cells; the 293T cell control contained 1.8 μg of protein from 293T cells transfected with only 20 μg of pHDV-EGFP. The A3G enzymatic activity observed in 1.8 μg of cell protein obtained from the transfected 293T cells was set to 100% (Table 3 and Fig. 3B). The relative A3G enzymatic activities observed in 0.225, 0.113 and 0.056 μg of the transfected 293T cell proteins were proportional to the amount of cell protein used in the assay. Linear regression analysis of these activities indicated an excellent fit and provided an R^2 value of 0.988, indicating that within this range, the enzymatic activity measurements were proportional to the amount of enzyme added (Fig. 3B, dashed box and Table 3). The A3G activity in the 293T cell control was only 1.5% of the 1.8 μg standard (Table 3).

Table 2
Relative A3G activity in NL43 and NL43Δ*vif* virions

Samples			No. of experiments	Relative A3G activity[a] (%) Average[b]±SE	*t* test[b] *P* value	[V-A3G$_{PBMC}$]/[V-A3G$_{2\ \mu g}$][c]
	Viruses	ng p24 CA				
293T cells cotransfected		1 ng	6	100.0±0.0		
with 2 μg of pcDNA-APO3G		0.5 ng	6	49.6±3.9		
		0.25 ng	6	16.6±1.1		
		0.125 ng	6	7.8±0.3		
293T cells	HIV-GFPΔ*vif*	2 ng	6	4.2±0.2		
PBMC donor #1	NL43Δ*vif*	2 ng	3	25.1±1.0	0.002	18.9%
	NL43	2 ng	4	6.6±0.3		
	Mock	Equal vol.[d]	3	7.4±0.9		
PBMC donor #2	NL43Δ*vif*	2 ng	3	27.7±2.0	<0.001	20.9%
	NL43	2 ng	4	6.2±0.1		
	Mock	Equal vol.	3	6.6±0.8		
PBMC donor #3	NL43Δ*vif*	2 ng	3	22.9±0.8	<0.001	17.3%
	NL43	2 ng	4	7.2±0.3		
	Mock	Equal vol.	3	5.2±0.3		
Average [V-A3G$_{PBMC}$]/[V-A3G$_{2\ \mu g}$] ratios						19.0%

[a] A3G activity in HIV-GFPΔ*vif* virions containing 1 ng of p24 CA that were produced from 293T cells transfected with 2 μg pcDNA-APO3G was set as 100%. The average cpm for samples containing 1 ng of p24 CA from 293T-derived virions was 42,881; the average cpm for samples without any HIV-GFP virions was 39.

[b] The *t* tests were performed to compare A3G activities in PBMC-derived virions with A3G activities in culture supernatants of mock-infected PBMCs.

[c] The ratio of A3G activity in NL43Δ*vif* virions produced from PBMCs [V-A3G$_{PBMC}$] to A3G activity in HIV-GFPΔ*vif* virions produced from 293T cells transfected with 2 μg of pcDNA-APO3G [V-A3G$_{2\ \mu g}$] was calculated by comparing the A3G activities in 2 ng of p24 CA from PBMC-derived NL43Δ*vif* virions with the A3G activity in 0.25 ng of p24 CA from 293T-derived virions, because the A3G activity in this 293T-derived virion sample was within the linear portion of the standard curve and closest to the A3G activity in the PBMC-derived virion samples. For example, the [V-A3G$_{PBMC}$]/[V-A3G$_{2\ \mu g}$] for PBMC donor #1-derived NL43Δ*vif* virions was calculated as follows: 25.1%/2 ng of p24 CA from the PBMC-derived virions ÷ 16.6%/0.25 ng of p24 CA from the 293T-derived virions ×100%=18.9%.

[d] Because the mock infected samples did not contain any detectable p24 CA (data not shown), volumes of these samples equivalent to the volume of culture supernatant from PBMCs producing NL43Δ*vif* virions and containing 2 ng of p24 CA were used in the SPA.

Next, the A3G activities present in 0.45 μg of protein from three activated PBMC preparations were compared to those present in the transfected 293T cells. The A3G activities in 0.45 μg of PBMC cell protein ranged from 26.9 to 32.3% of the activity in 1.8 μg of cell protein from 293T cells transfected with 2 μg of pcDNA-APO3G (Table 3 and Fig. 3B, green dot labeled with arrow). The A3G activities in the PBMCs were within the linear range of the assay (Fig. 3B). The ratio of A3G activity in PBMCs [C-A3G$_{PBMC}$] to the A3G activity in 293T cells transfected with 2 μg of pcDNA-APO3G [C-A3G$_{2\ \mu g}$] was determined using the A3G activities observed in 0.113 μg of the transfected 293T cell protein, because these activities were within the linear range and closest to the activities in the PBMCs. The average [C-A3G$_{PBMC}$]/[C-A3G$_{2\ \mu g}$] ratio was 26.9% (Table 3). Thus, the A3G activity in PBMCs was proportional to the A3G protein level in PBMCs (30%) as determined by quantitative immunoblotting (Fig. 3A).

Very little A3G protein in culture supernatants is associated with microvesicles

Microvesicles are protein-laden particles that have a density and size that is similar to HIV-1 virions (Bess et al., 1997; Gluschankof et al., 1997); as a result, microvesicles can be present in the density purified HIV-1 preparations (Trubey et al., 2003). To determine if the A3G enzymatic activity was associated with Δ*vif* virions rather than contaminating microvesicles, we digested HIV-GFPΔ*vif* virion preparations from human primary CD4$^+$T cells with subtilisin (Fig. 4, labeled Treated), a procedure that removes more than 95% of the contaminating microvesicle-associated proteins (Ott et al., 2000). We also performed mock-digestion on these HIV-GFPΔ*vif* virion preparations (labeled Mock) as a negative control. The results showed that the cytidine deaminase activities in subtilisin-digested samples were not significantly different from the mock-digested negative controls ($P=0.27$, *t* test). These results confirmed that very little of the A3G enzymatic activity was associated with microvesicles.

The A3G:Gag ratio and stoichiometry of A3G in PBMC-derived NL43Δvif virions

The results of the HPLC and SPA were used to calculate the A3G:Gag molar ratio in PBMC-derived NL43Δ*vif* virions as summarized in Table 4. Briefly, the A3G as a % of Gag in NL43Δ*vif* virions was calculated by multiplying the ratio of A3G activities in PBMC-derived and 293T-derived Δ*vif* virions ([V-A3G$_{PBMC}$]/[V-A3G$_{2\ \mu g}$]=19.0%) with the A3G as a % of Gag determined by HPLC in Δ*vif* virions produced from 293T cells transfected with 2 μg of pcDNA-APO3G ([V-A3G$_{2\ \mu g}$]/Gag=1.2%). The product of these two ratios provided a [V-A3G$_{PBMC}$]/Gag percentage of 0.228%, which is equivalent to an A3G:Gag molar ratio of 1:439 (Table 4).

Discussion

We combined three different experimental approaches – HPLC, quantitative immunoblotting analysis, and A3G cytidine

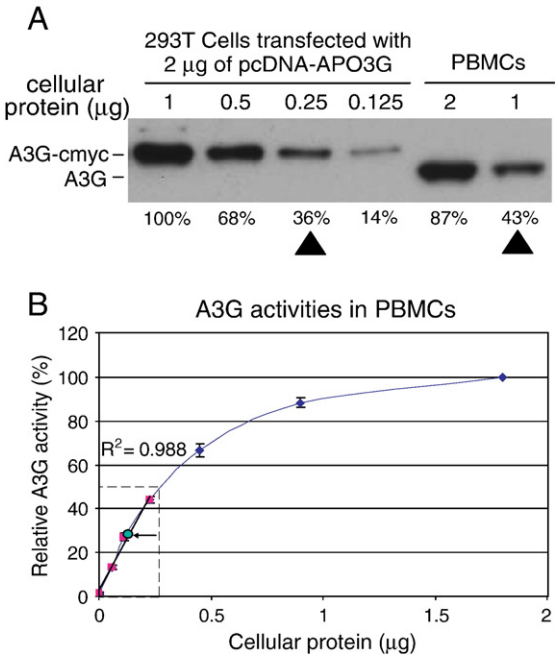

Fig. 3. Quantitative immunoblotting analysis and cytidine deaminase activities of A3G in PBMCs and 293T cells. (A) Quantitative immunoblotting analysis of A3G in extracts of PBMCs and 293T cells cotransfected with 2 μg of pcDNA-APO3G and 20 μg of pHDV-EGFP. The samples contained 1, 0.5, 0.25 and 0.125 μg of cellular protein harvested from the transfected 293T cells and 2 and 1 μg of cellular protein harvested from human PBMCs. The A3G protein was detected using an anti-A3G polyclonal antibody and the intensities of the bands were quantified by QuantityOne software (Bio-Rad). The intensity of the A3G band containing 1 μg of cellular protein of the transiently transfected 293T cells was set to 100%. The amount of A3G protein in PBMCs as a % of the amount of A3G in the transfected 293T cells was calculated using the bands with similar intensities (indicated by arrows) as follows: 43% A3G/1 μg of PBMC protein/ 36% A3G/0.25 μg of 293T cell protein × 100% = 30%. (B) Relative A3G activities in PBMCs. Standards contained 1.8, 0.9, 0.45, 0.225, 0.113 and 0.056 μg of cellular protein isolated from 293T cells transfected with 2 μg of pcDNA-APO3G. The linear portion of the standard curve is shown in a rectangle of dashed lines. Linear regression analysis indicating the best fit is shown as a solid black line, and the R^2 value is indicated. The average relative A3G activity of 0.45 μg cellular protein harvested from human PBMCs is shown as a green dot labeled with an arrow.

deaminase assay – to determine the A3G:Gag ratio in Δ*vif* virions. First, we determined A3G:Gag ratio in 293T-derived Δ*vif* virions using HPLC; second, we quantified the amounts of A3G protein in Δ*vif* virions by immunoblotting analysis; and third, we determined the ratio of A3G enzymatic activities in 293T- and PBMC-derived Δ*vif* virions. We then calculated the A3G:Gag ratio in PBMC-derived Δ*vif* virions by multiplying these two ratios to be approximately 1:439. The stoichiometry of Gag molecules per virion has been reported to be 1,400 (Zhu et al., 2003) and 5,000 (Briggs et al., 2004). The estimated A3G:Gag ratio of 1:439 and the reported range of 1,400 to 5,000 gag molecules per virion indicates that approximately 7±4 (3–11) A3G molecules are incorporated into each Δ*vif* virion.

Several observations indicate that the A3G enzymatic activities provide an accurate measurement of the amount of A3G protein incorporated in the PBMC-derived Δ*vif* virions. First, the cytidine deaminase assay showed an excellent linear range when serial dilutions of 293T-derived Δ*vif* virions containing A3G were analyzed. To maximize the precision of quantitation, we determined the A3G activities in PBMC-derived Δ*vif* virions within the linear range of the assay. Second, the A3G activity determinations (Average 19.0%) were in agreement with the A3G protein levels quantified by immunoblotting (22%). Third, the cytidine deaminase assay exhibited a low 4% background for 293T-derived Δ*vif* virions produced in the absence of pcDNA-APO3G, indicating that the assay has high sensitivity. Finally, wild type virions produced in the presence of Vif contained fourfold less A3G activity as expected, indicating that the assay can readily quantify Vif-induced reduction in A3G packaging.

The results of these studies demonstrate that virion incorporation of A3G is proportional to the amount of A3G expressed in the virion producing cells. The HPLC analysis indicated that the A3G:Gag ratio in virions produced from 293T cells transfected with 8 μg of pcDNA-APO3G was approximately 1:19. This result showed that when a HIV-1 vector:pcDNA-APO3G molar ratio of 1:0.8 is used to cotransfect 293T cells, the amount of A3G packaging is about 30-fold higher (74 to 264 A3G molecules/virion) than that present in virions produced from activated PBMCs. Thus, the conditions of the cotransfection assays could lead to a great excess of A3G incorporation into virions, potentially leading to inhibition of viral replication by mechanisms that are not relevant to natural HIV-1 infection.

Previous observations that Δ*vif* virions are replication defective in PBMCs (Madani and Kabat, 1998; Simon et al., 1998; von Schwedler et al., 1993), and our results that only 7±4 molecules of A3G are packaged in PBMC-derived virions indicate that virion incorporation of only a few molecules of A3G is sufficient to potently inhibit HIV-1 replication. Based on an average of seven A3G molecules per virion and a Poisson distribution, the frequency of virions lacking any A3G is approximately 0.09%. This expected low frequency of virions lacking A3G is more than sufficient to account for the observed 10-to 100-fold reduction in infectious titers of Δ*vif* virions in restricted cell lines (Borman et al., 1995; Gaddis et al., 2003).

In addition to A3G, APOBEC3F is also packaged into virions (Bishop et al., 2004; Liddament et al., 2004; Zheng et al., 2004). The relative contributions of these two antiviral proteins to inhibition of viral replication are not known. In future studies, it will be important to determine the stoichiometry of APOBEC3F to Gag in Δ*vif* virions.

Materials and methods

Plasmids

pHDV-EGFP (kindly provided by D. Unutmaz, Vanderbilt University) (Unutmaz et al., 1999), pcDNA-APO3G (kindly provided by K. Strebel, NIAID, National Institute of Health) (Kao et al., 2003), and pHCMV-G which expresses the vesiculostomatitis virus envelope glycoprotein G (VSV-G) were described previously (Yee et al., 1994).

Table 3
Relative A3G activity in cell lysates

Sample		Relative A3G activity[a] (%)				*t* test[b] *P* value	[C-A3G$_{PBMC}$]/[C-A3G$_{2\ \mu g}$][c]
Cell extracts	Total protein used (μg)	Exp 1	Exp 2	Exp 3	Average[b]±SE		
From 2 μg A3G transfected 293T	1.8	100.0	100.0	100.0	100.0±0.0		
	0.9	89.5	84.1	91.5	88.4±2.2		
	0.45	68.9	60.8	69.8	66.5±2.9		
	0.225	44.7	41.2	46.3	44.1±1.5		
	0.113	28.6	23.7	29.1	27.1±1.7		
	0.056		12.0	14.2	13.1±1.1		
293T	1.8	1.1	2.1	1.2	1.5±0.3		
PBMC donor #1	0.45	27.2	24.2	29.2	26.9±1.5	<0.001	24.7
PBMC donor #2	0.45	34.9	24.3	37.7	32.3±4.1	<0.001	29.7
PBMC donor #3	0.45	30.9	22.5	31.5	28.3±2.9	<0.001	26.1
Average [C-A3G$_{PBMC}$]/[C-A3G$_{2\ \mu g}$] ratio							26.9

[a] A3G activity in 1.8 μg of protein from 293T cells transfected with 2 μg pcDNA-APO3G was set as 100%. The average cpm for samples containing 1.8 μg of protein was 54,693; the average cpm for samples without any cell lysates was 57.

[b] The *t* tests were performed to compare A3G activities in PBMCs with A3G activities in mock-transfected 293T cells.

[c] The ratio of A3G activity in PBMCs [C-A3G$_{PBMC}$] to A3G activity in 293T cells transfected with 2 μg of pcDNA-APO3G [C-A3G$_{2\ \mu g}$] was calculated by comparing the A3G activities in 0.45 μg of PBMC cell lysates with the A3G activity in 0.113 μg of 293T cell lysate, because the A3G activity in this 293T sample was within the linear portion of the standard curve and closest to the A3G activity in the PBMC samples. For example, the [C-A3G$_{PBMC}$]/[C-A3G$_{2\ \mu g}$] for PBMC donor #1 was calculated as follows: 26.7%/0.45 μg of PBMC protein/27%/0.113 μg of 293T protein×100%=24.7%.

Cells

293T cells were maintained in Dulbecco's Modified Eagle's Medium with 10% calf serum as described previously (Svarovskaia et al., 2004). Human PBMCs were isolated from three different healthy donors through Histopaque (Sigma) gradients, activated by 2 μg/ml phytohemagglutinin for 3 days, and maintained in Rosswell Park Memorial Institute 1640 medium with 10% fetal calf serum and 200 units/ml of recombinant IL-2 (PeproTech) for 3 days. Cells were counted, washed with phosphate buffered saline (PBS), resuspended in PBS with 2% fetal calf serum, and the cell concentration was adjusted to 5×10^6 cells/ml prior to infection. Human CD4$^+$T cells were isolated from activated PBMCs using the CD4 Positive Isolation Kit (Dynal Biotech), resuspended in fresh medium with 200 units/ml of IL-2.

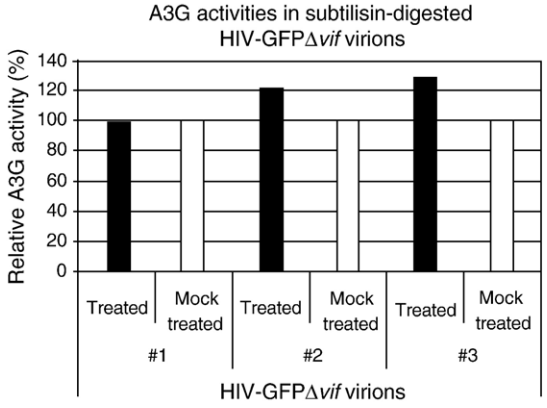

Fig. 4. A3G activities in subtilisin-digested virions. Human CD4$^+$T cells were obtained from three healthy donors and infected with HIV-GFPΔ*vif* virions. Virions harvested from these virion-producing CD4$^+$T cells were subjected to subtilisin- or mock-digestions, and subsequently analyzed by SPA. The incorporation of ^{3}H-dATP using the $(UUUA)_{10}$ template served as a positive control, and was set to 100%.

Transfection, virus purification, and infection

For determination of A3G stoichiometry in Δ*vif* virions produced from 293T cells by HPLC analysis, virions were produced from 293T cells that were transiently transfected with pHDV-EGFP (20 μg/100-mm-diameter plate) and pcDNA-APO3G (2, 4, or 8 μg/100-mm-diameter plate) using the calcium phosphate method (Sambrook and Russell, 2001). Viruses were harvested 48 h after transfection, filtered through 0.45-μm syringe filters (Corning Inc.), subjected to ultracentrifugation through 20% sucrose cushion (w/v) twice, and concentrated 100-fold by ultracentrifugation as described previously (Poon et al., 2002).

For determination of A3G stoichiometry in HIV-1 virions produced from human PBMCs, the cells were first infected with NL43 or NL43Δ*vif* viruses, which were produced by transient transfection of 293T cells with pNL43 and pNL43Δ*vif* expressing plasmids (20 μg/100-mm-diameter plate), respectively, using the calcium phosphate method. Viruses were

Table 4
Quantitation of A3G:Gag ratio in PBMC-derived Δ*vif* virions

Method	Comparison	Ratio	[V-A3G$_{PBMC}$][a]/Gag
HPLC	[V-A3G$_{2\ \mu g}$][b] / Gag[c]	1.2%	
Cytidine Deaminase Assay	[V-A3G$_{PBMC}$] / [V-A3G$_{2\ \mu g}$]	19.0%	
Calculation	[V-A3G$_{PBMC}$] / [V-A3G$_{2\ \mu g}$]× [V-A3G$_{2\ \mu g}$] / Gag		1.2%×19.0%=0.228% (1:439)

[a] [V-A3G$_{PBMC}$] is defined as average A3G molecules packaged in virions produced from human PBMCs infected with NL43Δ*vif* virions.

[b] [V-A3G$_{2\ \mu g}$] is defined as average number of A3G molecules packaged in HIV-GFPΔ*vif* virions produced from 293T cells transfected with 2 μg of pcDNA-APO3G and 20 μg of pHDV-EGFP.

[c] Gag is defined as average number of HIV-1 Gag molecules present in virions.

harvested 48 h after transfection, filtered through 0.45-μm syringe filters, and the amount of virus associated p24 CA was quantified by using the HIV-1 P24 ELISA Kit (Perkin Elmer). For each infection, viruses containing 100 ng of p24 CA were used to infect 2×10^6 cells for 24 h in the presence of polybrene (2 μg/ml). The infected cells were washed three times with PBS, incubated at 37 °C in medium containing 200 units/ml of IL-2 for 24 h, washed three times with PBS, and incubated for 3 days in fresh medium containing 200 units/ml of IL-2.

Culture medium containing viruses produced from the infected human PBMCs was filtered through 0.45-μm syringe filters, subjected to ultracentrifugation through sucrose cushion (20% w/v) three times, and concentrated 50-fold as described previously (Poon et al., 2002). The p24 CA quantities were determined in the concentrated virus by ELISA and viruses containing 2 ng of p24 were used for cytidine deamination assays.

Virions used for immunoblotting analysis and for generating a standard curve for cytidine deamination activity were produced from 293T cells transiently transfected with 2 μg of pcDNA-APO3G (0.5–8 μg/100-mm-diameter plate) and 20 μg of pHDV-EGFP by the calcium phosphate method, filtered, and concentrated 20-fold by ultracentrifugation. The p24 CA quantities in virion preparations were used to quantify the amounts of virion used in the cytidine deamination assays (Svarovskaia et al., 2004).

Cytoplasmic protein isolation and immunoblotting analysis

Cytoplasmic proteins from virus producing 293T and human PBMCs were isolated as described previously (Chen et al., 2002). The protein concentrations were measured by Bradford protein assay (Bio-Rad Laboratories). Cytoplasmic proteins and virus proteins were mixed with 1× SDS protein loading buffer, denatured at 94 °C for 4 min, separated by electrophoresis on a 12% Tris-Glycine Gel (Invitrogen), transferred to BioTrace PVDF Transfer Membrane (Pall Corporation), blocked by 5% milk or SuperBlock TBS (Pierce), probed with murine anti-c-Myc monoclonal antibody (Sigma) or murine monoclonal hA3G antibody (Immuno Diagnostics Inc.), and developed by the SuperSignal West Femto Maximum Sensitivity Kit (Pierce).

Subtilisin digestion of virions

HIV-GFPΔ*vif* virions harvested from *in vitro*-infected human PBMCs from three healthy donors were each divided into two parts and subjected to subtilisin or mock digestion, respectively. Subtilisin digestion of virions was carried out as previously described (Ott et al., 1995). Virions were reisolated by density centrifugation after protease digestion and the A3G activities present in the preparations were determined.

SPA for A3G activity

The assay was performed as described previously (Svarovskaia et al., 2004). A $(UUUA)_{10}$ oligonucleotide template was used in polymerization reaction and served as a positive control. The incorporation tritium-labeled deoxynucleotide triphosphates was quantified using Tricard 1600TR (Packard) liquid scintillation analyzer.

HPLC separation and analysis of viral proteins

Virus samples were disrupted in 8 M guanidine–HCl (Pierce,) with 50 mM dithiothreitol (Calbiochem,) and fractionated by HPLC to isolate viral proteins. HPLC was performed at a flow rate of 300 μl/min on 2.1×100 mm Poros R2/H narrow bore column (Applied Biosystems Inc.), using aqueous acetonitrile/trifluoroacetic acid solvents and a Shimadzu HPLC system equipped with LC-10AD pumps, SCL-10A system controller, CTO-10AC oven, FRC-10A fraction collector, and SPD-M10AV diode array detector. The gradient of buffer B (0.1% trifluoracetic acid in acetonitrile) was: 10−36.5%, 12 min; 36.5−37%, 4 min; 37−41%, 7 min; 41−70%, 12 min; and 70%, 5 min. The temperature was maintained at 55 °C during HPLC separation. Peaks were detected by UV absorption at 206 and 280 nm and analyzed by sequencing using an automated Applied Biosystems Inc. 477 Protein Sequencer and immunoblotting analysis. Quantitation of purified proteins was performed by amino acid analysis using a Hitachi L-8800 Amino Acid Analyzer.

Acknowledgments

We thank Eric Freed, Yeshitila Friew, Patricia Henry, and Jean Mbisa for critical reading of the manuscript and Anne Arthur for expert editorial assistance.

This research was supported in part by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research. This project has been funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under contract N01-CO-12400. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

References

Alce, T.M., Popik, W., 2004. APOBEC3G is incorporated into virus-like particles by a direct interaction with HIV-1 Gag nucleocapsid protein. J. Biol. Chem. 279 (33), 34083–34086.

Bess Jr., J.W., Gorelick, R.J., Bosche, W.J., Henderson, L.E., Arthur, L.O., 1997. Microvesicles are a source of contaminating cellular proteins found in purified HIV-1 preparations. Virology 230 (1), 134–144.

Bishop, K.N., Holmes, R.K., Sheehy, A.M., Davidson, N.O., Cho, S.J., Malim, M.H., 2004. Cytidine deamination of retroviral DNA by diverse APOBEC proteins. Curr. Biol. 14 (15), 1392–1396.

Bishop, K.N., Holmes, R.K., Malim, M.H., 2006. Antiviral potency of APOBEC proteins does not correlate with cytidine deamination. J. Virol. 80 (17), 8450–8458.

Borman, A.M., Quillent, C., Charneau, P., Dauguet, C., Clavel, F., 1995. Human immunodeficiency virus type 1 Vif-mutant particles from restrictive cells: role of Vif in correct particle assembly and infectivity. J. Virol. 69 (4), 2058–2067.

Briggs, J.A., Simon, M.N., Gross, I., Krausslich, H.G., Fuller, S.D., Vogt, V.M.,

Johnson, M.C., 2004. The stoichiometry of Gag protein in HIV-1. Nat. Struct. Mol. Biol. 11 (7), 672–675.
Cen, S., Guo, F., Niu, M., Saadatmand, J., Deflassieux, J., Kleiman, L., 2004. The interaction between HIV-1 Gag and APOBEC3G. J. Biol. Chem. 279 (32), 33177–33184.
Chen, J.J., Huang, J.C., Shirtliff, M., Briscoe, E., Ali, S., Cesani, F., Paar, D., Cloyd, M.W., 2002. CD4 lymphocytes in the blood of HIV(+) individuals migrate rapidly to lymph nodes and bone marrow: support for homing theory of CD4 cell depletion. J. Leukocyte Biol. 72 (2), 271–278.
Conticello, S.G., Harris, R.S., Neuberger, M.S., 2003. The Vif protein of HIV triggers degradation of the human antiretroviral DNA deaminase APOBEC3G. Curr. Biol. 13 (22), 2009–2013.
Gaddis, N.C., Chertova, E., Sheehy, A.M., Henderson, L.E., Malim, M.H., 2003. Comprehensive investigation of the molecular defect in vif-deficient human immunodeficiency virus type 1 virions. J. Virol. 77 (10), 5810–5820.
Gluschankof, P., Mondor, I., Gelderblom, H.R., Sattentau, Q.J., 1997. Cell membrane vesicles are a major contaminant of gradient-enriched human immunodeficiency virus type-1 preparations. Virology 230 (1), 125–133.
Harris, R.S., Liddament, M.T., 2004. Retroviral restriction by APOBEC proteins. Nat. Rev., Immunol. 4 (11), 868–877.
Harris, R.S., Bishop, K.N., Sheehy, A.M., Craig, H.M., Petersen-Mahrt, S.K., Watt, I.N., Neuberger, M.S., Malim, M.H., 2003. DNA deamination mediates innate immunity to retroviral infection. Cell 113 (6), 803–809.
Kao, S., Khan, M.A., Miyagi, E., Plishka, R., Buckler-White, A., Strebel, K., 2003. The human immunodeficiency virus type 1 Vif protein reduces intracellular expression and inhibits packaging of APOBEC3G (CEM15) a cellular inhibitor of virus infectivity. J. Virol. 77 (21), 11398–11407.
Klarmann, G.J., Chen, X., North, T.W., Preston, B.D., 2003. Incorporation of uracil into minus strand DNA affects the specificity of plus strand synthesis initiation during lentiviral reverse transcription. J. Biol. Chem. 278 (10), 7902–7909.
Layne, S.P., Merges, M.J., Dembo, M., Spouge, J.L., Conley, S.R., Moore, J.P., Raina, J.L., Renz, H., Gelderblom, H.R., Nara, P.L., 1992. Factors underlying spontaneous inactivation and susceptibility to neutralization of human immunodeficiency virus. Virology 189 (2), 695–714.
Lecossier, D., Bouchonnet, F., Clavel, F., Hance, A.J., 2003. Hypermutation of HIV-1 DNA in the absence of the Vif protein. Science 300 (5622), 1112.
Liddament, M.T., Brown, W.L., Schumacher, A.J., Harris, R.S., 2004. APOBEC3F properties and hypermutation preferences indicate activity against HIV-1 in vivo. Curr. Biol. 14 (15), 1385–1391.
Luo, K., Liu, B., Xiao, Z., Yu, Y., Yu, X., Gorelick, R., Yu, X.F., 2004. Amino-terminal region of the human immunodeficiency virus type 1 nucleocapsid is required for human APOBEC3G packaging. J. Virol. 78 (21), 11841–11852.
Madani, N., Kabat, D., 1998. An endogenous inhibitor of human immunodeficiency virus in human lymphocytes is overcome by the viral Vif protein. J. Virol. 72 (12), 10251–10255.
Mangeat, B., Turelli, P., Caron, G., Friedli, M., Perrin, L., Trono, D., 2003. Broad antiretroviral defence by human APOBEC3G through lethal editing of nascent reverse transcripts. Nature 424 (6944), 99–103.
Mariani, R., Chen, D., Schrofelbauer, B., Navarro, F., Konig, R., Bollman, B., Munk, C., Nymark-McMahon, H., Landau, N.R., 2003. Species-specific exclusion of APOBEC3G from HIV-1 virions by Vif. Cell 114 (1), 21–31.
Marin, M., Rose, K.M., Kozak, S.L., Kabat, D., 2003. HIV-1 Vif protein binds the editing enzyme APOBEC3G and induces its degradation. Nat. Med. 9 (11), 1398–1403.
Newman, E.N., Holmes, R.K., Craig, H.M., Klein, K.C., Lingappa, J.R., Malim, M.H., Sheehy, A.M., 2005. Antiviral function of APOBEC3G can be dissociated from cytidine deaminase activity. Curr. Biol. 15 (2), 166–170.
Ott, D.E., Coren, L.V., Johnson, D.G., Sowder II, R.C., Arthur, L.O., Henderson, L.E., 1995. Analysis and localization of cyclophilin A found in the virions of human immunodeficiency virus type 1 MN strain. AIDS Res. Hum. Retroviruses 11 (9), 1003–1006.
Ott, D.E., Coren, L.V., Johnson, D.G., Kane, B.P., Sowder II, R.C., Kim, Y.D., Fisher, R.J., Zhou, X.Z., Lu, K.P., Henderson, L.E., 2000. Actin-binding cellular proteins inside human immunodeficiency virus type 1. Virology 266 (1), 42–51.
Pathak, V.K., Temin, H.M., 1990. Broad spectrum of in vivo forward mutations, hypermutations, mutational hotspots in a retroviral shuttle vector after a single replication cycle: deletions and deletions with insertions. Proc. Natl. Acad. Sci. U.S.A. 87 (16), 6024–6028.
Poon, D.T., Chertova, E.N., Ott, D.E., 2002. Human immunodeficiency virus type 1 preferentially encapsidates genomic RNAs that encode Pr55(Gag): functional linkage between translation and RNA packaging. Virology 293 (2), 368–378.
Sambrook, J., Russell, D.W., 2001. 3rd Edition. Mol. Cloning: Lab. Man., vols. 3.3. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
Schafer, A., Bogerd, H.P., Cullen, B.R., 2004. Specific packaging of APOBEC3G into HIV-1 virions is mediated by the nucleocapsid domain of the gag polyprotein precursor. Virology 328 (2), 163–168.
Sheehy, A.M., Gaddis, N.C., Choi, J.D., Malim, M.H., 2002. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. Nature 418 (6898), 646–650.
Simon, J.H., Gaddis, N.C., Fouchier, R.A., Malim, M.H., 1998. Evidence for a newly discovered cellular anti-HIV-1 phenotype. Nat. Med. 4 (12), 1397–1400.
Stopak, K., de Noronha, C., Yonemoto, W., Greene, W.C., 2003. HIV-1 Vif blocks the antiviral activity of APOBEC3G by impairing both its translation and intracellular stability. Mol. Cell 12 (3), 591–601.
Svarovskaia, E.S., Xu, H., Mbisa, J.L., Barr, R., Gorelick, R.J., Ono, A., Freed, E.O., Hu, W.S., Pathak, V.K., 2004. Human apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G) is incorporated into HIV-1 virions through interactions with viral and nonviral RNAs. J. Biol. Chem. 279 (34), 35822–35828.
Trubey, C.M., Chertova, E., Coren, L.V., Hilburn, J.M., Hixson, C.V., Nagashima, K., Lifson, J.D., Ott, D.E., 2003. Quantitation of HLA class II protein incorporated into human immunodeficiency type 1 virions purified by anti-CD45 immunoaffinity depletion of microvesicles. J. Virol. 77 (23), 12609–12699.
Unutmaz, D., KewalRamani, V.N., Marmon, S., Littman, D.R., 1999. Cytokine signals are sufficient for HIV-1 infection of resting human T lymphocytes. J. Exp. Med. 189 (11), 1735–1746.
Vartanian, J.P., Meyerhans, A., Asjo, B., Wain-Hobson, S., 1991. Selection, recombination, G-A hypermutation of human immunodeficiency virus type 1 genomes. J. Virol. 65 (4), 1779–1788.
von Schwedler, U., Song, J., Aiken, C., Trono, D., 1993. Vif is crucial for human immunodeficiency virus type 1 proviral DNA synthesis in infected cells. J. Virol. 67 (8), 4945–4955.
Yee, J.K., Friedmann, T., Burns, J.C., 1994. Generation of high-titer pseudotyped retroviral vectors with very broad host range. Methods Cell Biol. 43 (Pt. A), 99–112.
Yu, X., Yu, Y., Liu, B., Luo, K., Kong, W., Mao, P., Yu, X.F., 2003. Induction of APOBEC3G ubiquitination and degradation by an HIV-1 Vif–Cul5–SCF complex. Science 302 (5647), 1056–1060.
Zennou, V., Perez-Caballero, D., Gottlinger, H., Bieniasz, P.D., 2004. APOBEC3G incorporation into human immunodeficiency virus type 1 particles. J. Virol. 78 (21), 12058–12061.
Zhang, H., Yang, B., Pomerantz, R.J., Zhang, C., Arunachalam, S.C., Gao, L., 2003. The cytidine deaminase CEM15 induces hypermutation in newly synthesized HIV-1 DNA. Nature 424 (6944), 94–98.
Zheng, Y.H., Irwin, D., Kurosu, T., Tokunaga, K., Sata, T., Peterlin, B.M., 2004. Human APOBEC3F is another host factor that blocks human immunodeficiency virus type 1 replication. J. Virol. 78 (11), 6073–6076.
Zhu, P., Chertova, E., Bess Jr., J., Lifson, J.D., Arthur, L.O., Liu, J., Taylor, K.A., Roux, K.H., 2003. Electron tomography analysis of envelope glycoprotein trimers on HIV and simian immunodeficiency virus virions. Proc. Natl. Acad. Sci. U.S.A. 100 (26), 15812–15817.